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14. ABSTRACT

In mammals and other higher eukaryotes, SRA-RING proteins are essential for global maintenance of DNA methylation. This research project focuses on the discovery of new ubiquitination targets for the Arabidopsis SRA-RING protein VIM1 and the human SRA-RING protein UHRF1. Additionally, it will closely examine the methylcytosine-binding specificity of UHRF1, with a specific focus on non-CpG contexts. The proposed work is ongoing, and so far the major accomplishments include creation of relevant plant lines, development of in vitro assays, and profiling of mRNA expression in null mutants.

15. SUBJECT TERMS
DNA methylation, UHRF1, VIM1, ubiquitination, epigenetics, chromatin

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INTRODUCTION

DNA methylation is an important regulator of genome function, and disruptions in DNA methylation play a role in many types of cancers. Several studies have identified specific DNA methylation patterns as prognostic markers for breast cancer[1-4]. In mammals and other higher eukaryotes, SRA-RING proteins are essential for global maintenance of DNA methylation[5,6]. These proteins also regulate cellular processes relevant to breast cancer pathology, including gene expression and the cell cycle[7-10]. Each protein in the SRA-RING family contains a methylcytosine-binding SRA (SET- and RING-associated) domain, a PHD domain, and one or more RING domains. Studies in mammalian cell lines have shown that the RING domain of ICBP90/UHRF1, a SRA-RING protein that is often misregulated in cancers, can target core histones or DNMT1 for ubiquitination[11,12]. Although these studies have provided valuable insight into the function of UHRF1 in epigenetic regulation and heterochromatin structure, they have not exhaustively considered other potential substrates for UHRF1 ubiquitin ligase activity. A significant portion of my research project focuses on the discovery of new ubiquitination targets for the Arabidopsis SRA-RING protein VIM1, a UHRF1 ortholog with a similar domain structure and comparable roles in epigenetic regulation (Fig 1). I will follow up on these studies with in vitro experiments involving UHRF1 aimed at further understanding the substrates and specificities of its RING and SRA domains. This research will provide details on fundamental epigenetic mechanisms that are central to the molecular pathology of breast cancer.

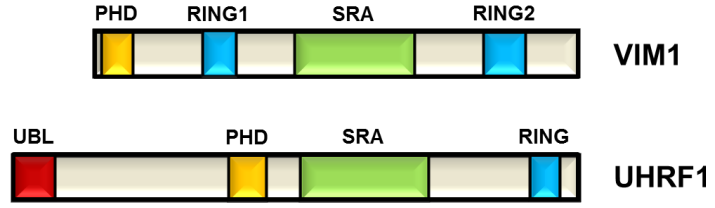

Figure 1. Arabidopsis VIM1 and human UHRF1 proteins share a similar domain structure, with each containing a PHD domain, an SRA domain, and at least one RING domain.

BODY

Here, I discuss training and research accomplishments associated with a revised Statement of Work (SOW) submitted as Amendment P00004 to project W81XWH-10-1-0080, effective August 11, 2011.

Training Plan

Task 1: Complete coursework that will provide a background in the molecular biology of breast cancer

1. "Regulation of Cell Proliferation, Senescence, and Death," which covers topics in the cell cycle and signal transduction, including lectures on oncogenesis (Months 2 – 5)
2. "Cellular and Molecular Pharmacology," which surveys receptor mechanisms and signaling pathways, including topics in drug-receptor interactions, gene expression, and chemotherapy (Months 9 – 12)

My graduate coursework has included "The Molecular Basis of Human Disease," which covered several weeks' worth of material specifically related to the molecular and epigenetic basis of cancer; "Epigenetics," which provided an understanding of basic epigenetic mechanisms as well as their significance in cancer and disease; "Genomes as Chromosomes," a minicourse that enhanced my understanding of genome function and also covered cancer-related topics; and "The Nucleus," which covered chromatin structure, transcription, RNA processing, and other nuclear processes important for understanding the molecular basis of cancer. In addition, I have taken many courses that provide a basic knowledge of biochemistry, molecular biology, and genetics that is essential for a successful research career. My coursework has gone a different direction than I anticipated when writing my SOW, and I have not taken either of the two courses that I proposed. However, I am confident that the coursework I have completed provides me with a strong academic background for breast cancer research.

Task 2: Regularly attend meetings with Cornell laboratories that study topics related to breast cancer and epigenetics

1. Monthly journal club meetings at the Center for Vertebrate Genomics (CVG) which discusses papers on cancer and is attended by members of the Nikitn and Weiss labs, which study molecular processes involved in cancer pathogenesis (Monthly, Months 1-36)
2. Monthly meetings with the Cornell Epigenetics and Chromatin Collective (EpiC), which is attended by numerous epigenetics researchers on campus and regularly features presentations on breast cancer-related topics

Since I have transitioned to a non-vertebrate model system, I have not been attending the monthly CVG meetings. I had been regularly attending the EpiC monthly meetings, but as of 2011 this group is no longer active on the Cornell campus. I continually watch for new opportunities to attend meetings with other Cornell University cancer researchers, and I attend as many breast cancer-related seminars as possible.

Task 3: Interact with Cornell University breast cancer researchers at on-campus events

1. Present at the annual CVG symposium, where I will interact with members of the CVG and discuss my research with other scientists in the field of cancer research (Yearly, Months 1 – 36)
2. Attend the bi-annual Cancer and Environment Forum held by the Breast Cancer and Environmental Risk Factors program (Twice yearly, Months 1 – 36)
3. Regularly discuss research progress with Scott Coonrod, who is a co-mentor of the research project

Since my transition to Arabidopsis as a model system, I have not recently been active with the Center for Vertebrate Genomics. The BCERF program is also no longer active at Cornell. However, I continue to meet regularly with Scott Coonrod, who is a co-mentor of my research project and a member of my thesis committee. Dr. Coonrod is a fellow epigenetics researcher who has offered great insight in our discussions, and I look forward to his continuing support.

Task 4: Present at national conferences on epigenetics and breast cancer (Yearly, Months 1 – 36)

1. The American Association for Cancer Research annual meeting
2. DOD Breast Cancer Research Program's Era of Hope meeting
3. FASEB Summer Research Conferences

Breast cancer and epigenetics-related conferences I have attended include: 1) The FASEB Summer Research Conference on Biological Methylation: From DNA to Histones, June 6-11, 2010 in Carefree, Arizona; 2) The DOD Breast Cancer Research Program's Era of Hope meeting, August 2-5, 2011 in Orlando, Florida; and 3) The Keystone Joint Symposium on Chromatin Dynamics and Epigenomics, January 17-22, 2012, in Keystone, Colorado. At each of these meetings, I have given poster presentations and had valuable exchanges with cancer and epigenetics researchers.

Task 5: Develop laboratory and bioinformatics skills for genomic analyses (Months 24-36)

Though this task was not originally proposed in my Statement of Work, it has substantially advanced my proficiency in skills that will support my future research career. The ability to perform genomic studies is becoming increasingly relevant to epigenetics and breast cancer research, as numerous studies have uncovered global misregulation of genes and epigenetic marks in breast cancer. Transcriptional profiling and mapping of epigenetic modifications are among the future applications of my training in genomics. I have accomplished the following:

1. Learn techniques for construction of strand-specific RNA-seq libraries for analysis on Illumina sequencing platforms (Months 24-26)
2. Acquire proficiency with command-line tools for RNA-seq analysis, including TopHat, Cufflinks, and DESeq (Months 28-36)
3. Take courses in programming and statistics, including: "Perl for Biologists" workshop offered by the Cornell Biology Service Unit, March 6-June 12, 2013; and "Mathematical Biostatistics Bootcamp 1," an intensive introduction to statistics offered online by Brian Caffo of Johns Hopkins University, April 16-May 31, 2013

Research Plan

Task 1: Investigate the role of the VIM1 RING domain in epigenetic regulation in *Arabidopsis thaliana*

1. Identify substrates for VIM1 E3 ubiquitin ligase activity using a comparative proteomics approach in wild-type plants and RING domain mutants (Months 1 - 18)

To understand the contributions of individual VIM1 domains in epigenetic regulation, I have transformed *vim1 -/-* mutants with T-DNA constructs for expression of the *VIM1* gene under its native promoter. Through site-directed mutagenesis, point mutations have been made in zinc-chelating residues of one or both of the VIM1 RING domains in these expression constructs. Thus, the only form of VIM1 expressed in these plant lines will be a version containing point mutations that should abolish RING domain activity. Additionally, since the function of the VIM1 PHD domain is also poorly understood, I have created a PHD point mutant line for further study (Fig. 2).

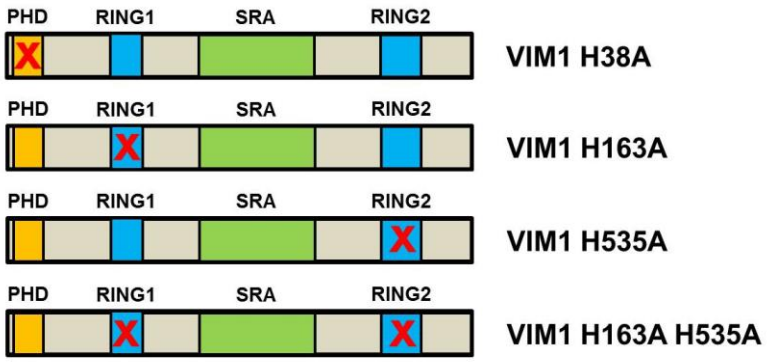

Figure 2. VIM1 point mutants expressed in a vim1 -/- background for comparative proteomics experiments. Each domain targeted for site-directed mutagenesis is indicated with a red X.

I intend to use these plant lines in future studies that compare protein levels between wild type plants and the VIM1 RING domain mutants using iTRAQ (isobaric tag for relative and absolute quantitation). In this experiment, nuclear protein from wild-type plants and *vim1 -/-* plants expressing the *VIM1* RING mutant constructs will be digested and labeled with different chemical tags. The samples will then be pooled and analyzed via nano liquid chromatography followed by tandem mass spectrometry. The two tags will generate distinct reporter ions when fragmented, allowing for relative quantitation of proteins between the samples. Significant abundance of a protein in the RING mutant plants relative to wild type may suggest that VIM1 participates in turnover of that protein, consistent with the fact that ubiquitination often targets proteins for proteasome-mediated degradation.

These experiments are still in progress, and there are no reportable results as of the end of the project period. However, creation of the relevant plant lines has been an important step toward completing this objective, which will remain a focus of future work.

2. Confirm ubiquitination targets using an in vitro assay containing purified recombinant VIM1 (Months 1 – 36)

Though I have not yet completed the proteomic analysis described above, I have performed additional biochemical analyses that provide insight on the functional importance of the VIM1 PHD domain and suggest that histones are possible VIM1 ubiquitination targets. PHD domains often act as “readers” of epigenetic marks through specific interactions with modified histones. Consequently, I used commercially available modified histone peptide arrays to screen for binding interactions between the VIM1 PHD domain and a wide variety of histone marks. These experiments have identified methyl marks on histone H3 lysine 27 as binding targets for the VIM1 PHD domain, suggesting that VIM1 may recognize and colocalize with these marks in the genome (Fig. 3). The PHD domain

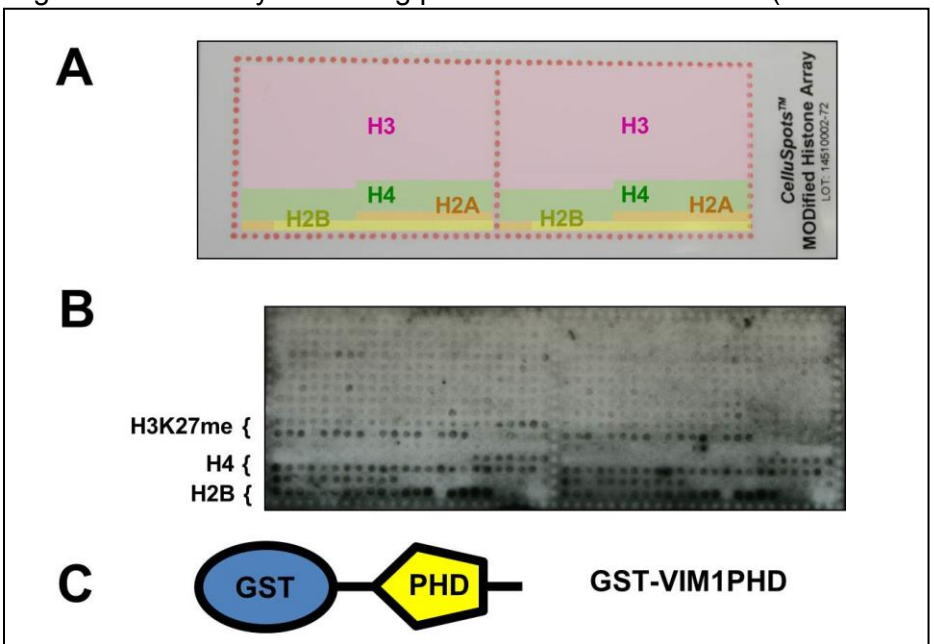

Figure 3. The VIM1 PHD domain interacts with histones H2B, H4, and H3 methylated on lysine 27. A) Diagramof histone peptides on the peptide array, which is commercially available from Active Motif. B) Reperesentative image indicating VIM1 PHD interactions with histone peptides. C) Schematic representation of the GST-tagged VIM1 partial protein used to probe the array.

also interacts with histones H4 and H2B, though these interactions appear to be less specific to certain modifications. Since VIM1 directly interacts with three of the four core histones via its PHD domain in vitro, histones are plausible targets for ubiquitination by its RING domains.

I have developed an in vitro ubiquitination system that will be useful for assaying the ubiquitination of histones and other candidate substrates. In these assays, the required E1, E2, and E3 enzymes are combined in vitro under appropriate buffer conditions with other necessary components required for ubiquitination, including ubiquitin and ATP. Commercially available yeast UBE1, purified recombinant GST-UBC8, and purified recombinant GST-VIM1 act as the E1, E2, and E3 respectively. Using this system, I have been able to recapitulate results previously reported by another laboratory, demonstrating that VIM1 has E3 ubiquitin ligase activity in vitro[13]. In addition, I have used partial recombinant fragments of VIM1 to show that each of the two individual RING domains has activity (Fig. 4).

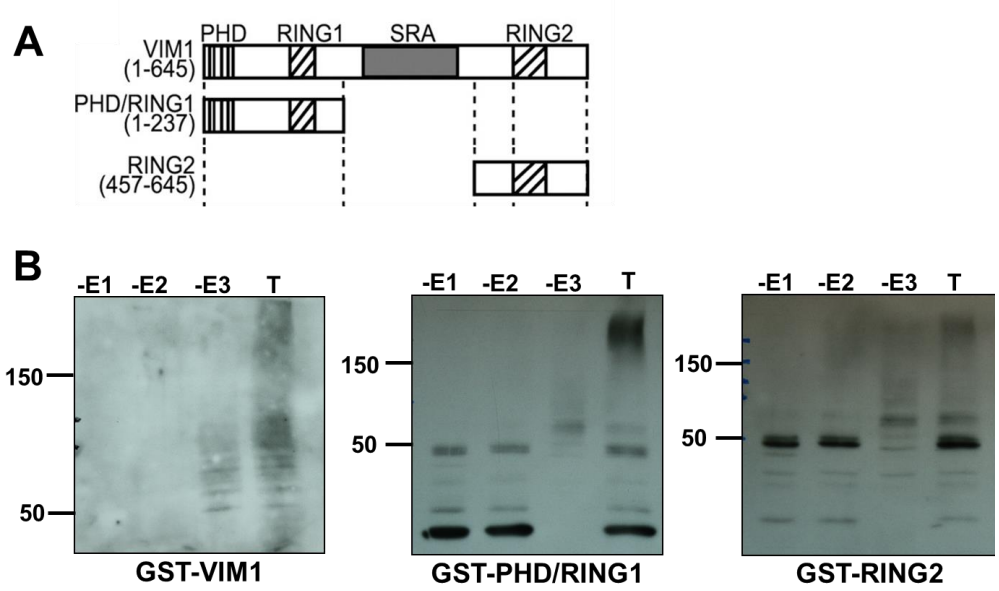

Figure 4. VIM1 RING domains have E3 ubiquitin ligase activity *in vitro*. A) Schematic representation of full-length and partial VIM1 proteins used in the ubiquitination assay. Length of each protein in amino acids is indicated. B) *in vitro* ubiquitination reactions lacking E1 enzyme (-E1), E2 enzyme (-E2), E3 enzyme (-E3), or containing all essential enzymes (T). The E3 protein used in each experiment is labeled at the bottom of the images. Ubiquitination reactions were run on a polyacrylamide gel, then immunoblotting was done against ubiquitin. The high molecular weight smear in the complete (T) reactions indicates the synthesis of polyubiquitin chains in vitro.

3. Assess the effects of RING domain mutations on DNA methylation and heterochromatin structure in vivo (Months 1 - 36)

I used the plant lines expressing the point mutants described in Task 1, section 1 to determine the relationship between VIM1 ubiquitin ligase activity and other epigenetic processes, such as maintenance of DNA methylation and heterochromatin structure. Along with a summer undergraduate intern, Elena Cravens, I examined the DNA methylation status of centromeric repeat sequences in these lines. Specifically, we digested genomic DNA extracted from the plant lines with HpaII, a methylation-senstive enzyme, then visualized the digestion pattern using southern blots against a 180-base pair centromere repeat. While fully methylated repeats appear undigested in this assay, regular cutting of unmethylated repeats yields a ladder-like digestion pattern in hypomethylated samples. Our results suggest that neither of the two RING domains is independently required for maintenance of methylation at the centromeres, but that the two domains may function cooperatively (Figure 5A). Neither the H163A mutation in the N-terminal RING domain nor the H535A mutation in the C-terminal RING domain is sufficient to significantly reduce methylation at the centromeric repeats. However, combining the two mutations within a single VIM1 expression construct results in a HpaII digestion pattern similar to that seen in a *vim1* null mutant. This result suggests that the two domains ubiquitinate a common target, and that this activity is required for maintenance of centromeric repeat methylation. Expression analysis of the VIM1 point mutant transgenes in each line verified that transgenes were expressed at levels comparable to that of VIM1 in wild-type plants (Figure 5B), providing confirmation that the observed methylation phenotypes are not due to significant over- or under-expression of the transgenes.

Interestingly, these experiments also revealed a role for the VIM1 PHD domain in maintaining centromeric DNA methylation. An H38A mutation in the VIM1 PHD domain resulted in hypomethylation of the centromere repeats (Figure 5A). Together with the results from Task 1, section 2 indicating that the PHD interacts with histone N-terminal tails, this outcome suggests that an interaction between the VIM1 PHD domain and modified histones are mechanistically important for maintenance of DNA methylation.

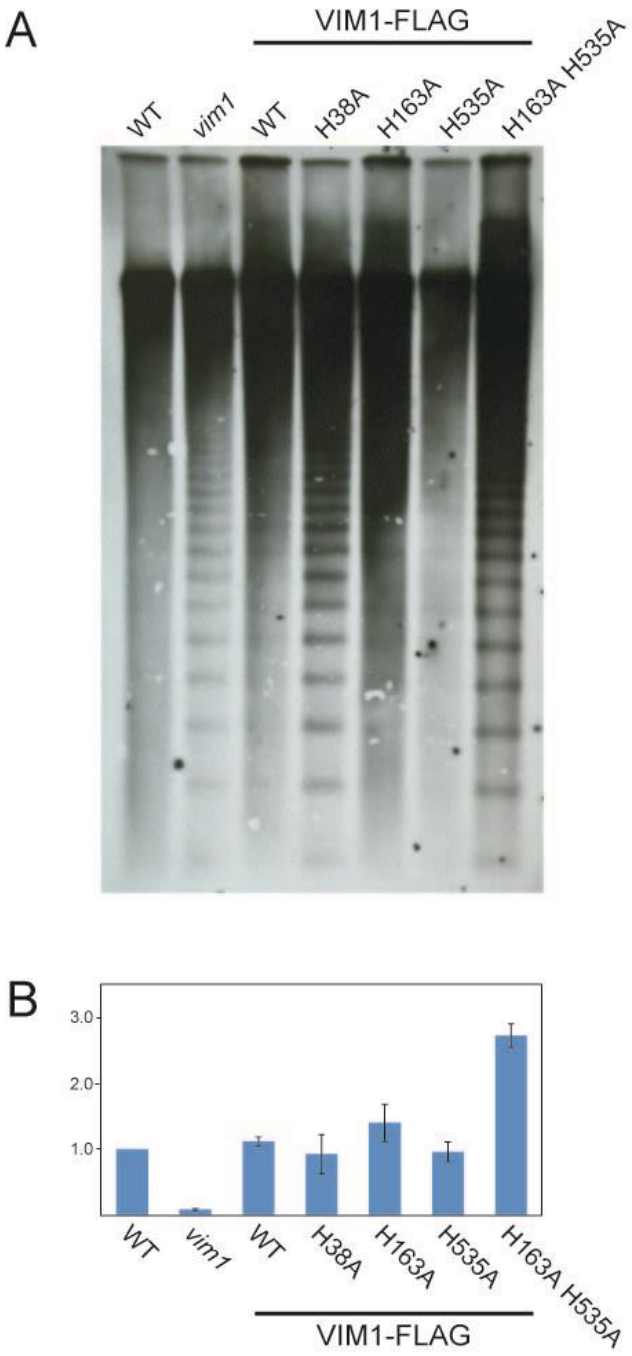

Figure 5. The VIM1 N- and C-terminal RING domains function differently in the maintenance of DNA methylation at centromere repeats. Genomic DNA was digested with HpaII or MspI as indicated, then probed in southern blots with a 180-bp centromere repeat probe. Col WT and Col *vim1* are shown as methylated and hypoethylated controls, respectively. The remaining lanes show samples from different Col *vim1* individuals expressing WT or point mutant VIM1-FLAG.

Task 2: Determine the *in vitro* specificities of the human UHRF1 RING and SRA domains

1. Examine UHRF1 E3 ligase activity on human homologs of VIM1 substrates in vitro (Months 18 – 36)

These experiments are dependent on the outcome of the work proposed in Task 1, section 1. As that work is still in progress, I have not yet been able to initiate an investigation of UHRF1 ubiquitination activity in vitro.

2. Determine the methylcytosine-binding activity of the UHRF1 SRA domain using electrophoretic mobility shift assays (EMSA) (Months 1-24)

Although it has been previously reported that the mouse homolog of UHRF1 specifically recognizes methylcytosine in CpG contexts[5], I proposed to determine whether the human protein has the same specificity. The prospect of UHRF1 binding methylcytosine in non-CpG contexts was particularly intriguing, since the existence of non-CpG methylation has recently been reported in humans[14,15]. I worked on these experiments together with Erika Hughes, a research technician in the Richards lab. Together, we cloned and purified several UHRF1 partial proteins containing the SRA domain, and created several methylated double-stranded oligonucleotides for use as substrates (Fig. 6).

Task 3: Compare gene expression profiles between *vim1 vim2 vim3* mutants and *met1* mutants

The Arabidopsis VIM proteins, including VIM1, putatively function in a complex with the DNA methyltransferase MET1, a homolog of mammalian DNMT1, to regulate cytosine methylation in the CG dinucleotide context (Figure 7). As supporting evidence of this mechanism, the reduction in CG methylation seen in *vim1 vim2 vim3* mutants resembles the methylation pattern observed in a *met1* mutant[16]. However, it is unknown whether VIM proteins, perhaps through their PHD or RING activity, participate in epigenetic mechanisms aside from maintenance of DNA methylation. If so, it might be expected that VIM proteins

transcriptionally regulate additional targets besides those regulated by MET1. To test this hypothesis, I used an RNA-seq approach to profile gene expression in vim1 vim2 vim3 and met1 mutants. This experiment was not part of the research originally proposed in my Statement of Work. However, it fits into the overall scope of the proposed work by potentially providing new information on the functions of the PHD and RING domains in epigenetic regulation of gene expression.

A strand-specific protocol was followed to prepare Illumina RNA-seq libraries from wild-type, *met1*, and *vim1 vim2 vim3* inflorescence tissue in triplicate[18]. 50 bp single-end reads were sequenced at Weill Cornell Medical College on the Illumina Hi-Seq 2000 platform. The reads were mapped using TopHat, then differential expression was analyzed between samples using the R package DESeq[19,20].

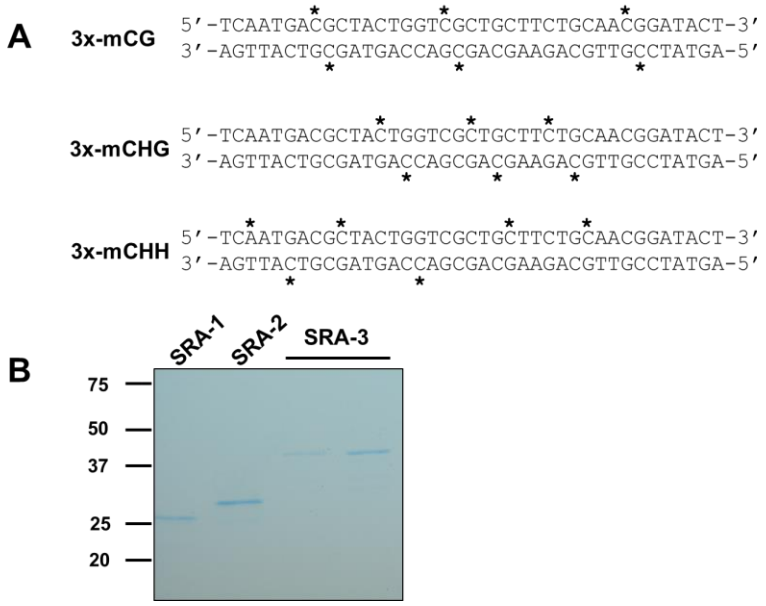

Figure 6. Reagents generated for EMSA experiments. A) Double-stranded oligonucleotide substrates bearing CG, CHG, or CHH substrates. The double-stranded oligos are identical aside from placement of the methyl groups. B) Purified recombinant His-tagged SRA domains. SRA-1 and SRA-2 are the same SRA domains used in previously published literature from other groups[5,17]. SRA-3 is an extended version.

Our analysis revealed that thousands of transcripts were up- or down-regulated in *met1* and *vim1 vim2 vim3* relative to wild-type plants. Importantly, the set of transcripts significantly differentially expressed in *met1* mutants relative to wild-type overlapped almost entirely with those misregulated in *vim1 vim2 vim3* mutants (Figure 8). This result provides strong evidence that MET1 and the VIM proteins function in a complex co-regulate the same set of transcripts, as hypothesized. Our analysis uncovered a relatively small set 192 of potential VIM-specific targets, presenting the intriguing possibility that VIM proteins could be involved in regulating genes independently of MET1. However, follow-up with RT-qPCR expression analysis failed to confirm that any of these transcripts were misregulated in vim1 vim2 vim3 mutants, suggesting that they are false positives resulting from the RNA-seq analysis (data not shown). In future experiments, I will further explore the potential interaction between VIM proteins and MET1 using in vitro pull-down assays. Combined with the RNA-seq results identifying co-regulated transcripts, evidence of a direct interaction between MET1 and VIM proteins would offer convincing support for the two proteins acting in a complex to regulate DNA methylation.

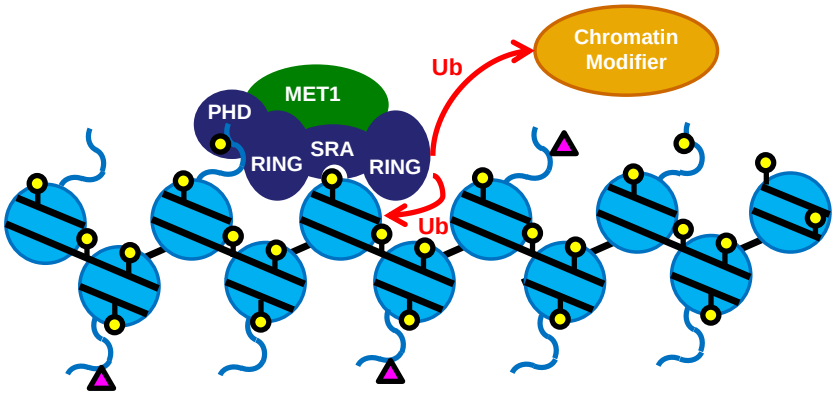

Figure 7. Model for the co-regulation of DNA methylation by a VIM-MET1 complex. The complex is putatively recruited to target genomic regions through interactions between the VIM SRA domain and hemi-methylated CG sites, as well as interactions between the VIM PHD domain and histone N-terminal tails.

KEY RESEARCH ACCOMPLISHMENTS

- Creation of plant lines expressing VIM1 RING point mutants under a native promoter in a *vim1* -/- background. These will be useful for two of the experiments discussed in Task 1.
- Discovery of direct interactions between the VIM1 PHD domain and specific histones, including H2B, H4, and H3 with lysine 27 methylation

- Development of an in vitro ubiquitination assay for confirmation of candidate VIM1 substrates
- Confirmation of VIM1 E3 ubiquitin ligase activity in vitro, and demonstration that each of the two VIM1 RING domains independently has activity
- Confirmation that the VIM1 RING domains and PHD domain are required for maintenance of centromeric DNA methylation
- Purification of UHRF1 SRA domains and generation of oligonucleotide substrates for EMSA experiments

REPORTABLE OUTCOMES

- Poster presentation, "In vitro mechanism of UHRF1 methylcytosine binding," at Era of Hope Conference, August 5 2011
- Poster presentation, "Binding specificity of the Arabidopsis VIM1 PHD domain," at Keystone Symposium on Chromatin Dynamics, January 20, 2012
- Manuscript in preparation, "Functional analysis of VIM1 PHD and RING domains in the maintenance of DNA methylation"

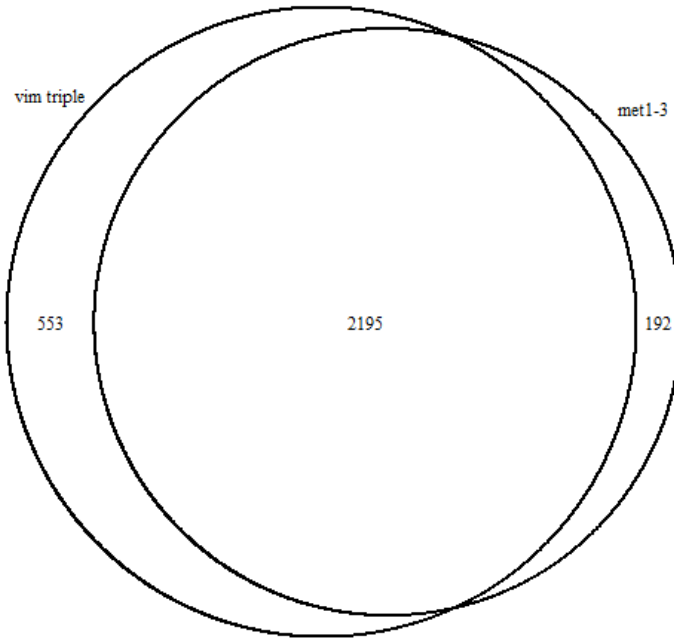

Figure 8. mRNA regulatory targets of VIM proteins (labeled "vim triple") and MET1 (labeled "met1-3") overlap extensively. Our analysis identified 2195 transcripts misregulated in both *vim1 vim2 vim3* mutants and *met1* mutants.

CONCLUSIONS

The outcomes of this project have already provided useful insights into the functions of PHD and RING domains in VIM1, an ortholog of the human UHRF1 protein. In particular, we have demonstrated that these domains are required for maintenance of DNA methylation at centromere repeats. We have also identified a set of VIM mRNA regulatory targets and shown that they are highly similar to the set of MET1 targets. Understanding how these proteins function on the molecular level can eventually lead to the development of new epigenetically based breast cancer therapies and diagnostic tools. Future efforts will focus on the proposed proteomics experiments for identification of new ubiquitinated substrates, confirmation of these substrates using in vitro assays, and optimization of the EMSA protocol.

REFERENCES

1. Novak, P. et al. Agglomerative epigenetic aberrations are a common event in human breast cancer. *Cancer Res* **68**, 8616-8625 (2008).
2. Hartmann, O. et al. DNA methylation markers predict outcome in node-positive, estrogen receptor-positive breast cancer with adjuvant anthracycline-based chemotherapy. *Clin. Cancer Res* **15**, 315-323 (2009).
3. Sinha, S. et al. Frequent alterations of hMLH1 and RBSP3/HYA22 at chromosomal 3p22.3 region in early and late-onset breast carcinoma: clinical and prognostic significance. *Cancer Sci* **99**, 1984-1991 (2008).
4. Rønneberg, J.A. et al. GSTP1 promoter haplotypes affect DNA methylation levels and promoter activity in breast carcinomas. *Cancer Res* **68**, 5562-5571 (2008).
5. Bostick, M. et al. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science* **317**, 1760-1764 (2007).
6. Woo, H.R., Pontes, O., Pikaard, C.S. & Richards, E.J. VIM1, a methylcytosine-binding protein required for centromeric heterochromatinization. *Genes Dev* **21**, 267-277 (2007).
7. Wu, J. et al. Identification and functional analysis of 9p24 amplified genes in human breast cancer. *Oncogene* (2011).doi:10.1038/onc.2011.227
8. Tien, A.L. et al. UHRF1 depletion causes a G2/M arrest, activation of DNA damage response and apoptosis. *Biochem. J* **435**, 175-185 (2011).

9. Daskalos, A. et al. UHRF1-mediated tumor suppressor gene inactivation in nonsmall cell lung cancer. *Cancer* **117**, 1027-1037 (2011).
10. Alhosin, M. et al. Down-regulation of UHRF1, associated with re-expression of tumor suppressor genes, is a common feature of natural compounds exhibiting anti-cancer properties. *J. Exp. Clin. Cancer Res* **30**, 41 (2011).
11. Du, Z. et al. DNMT1 stability is regulated by proteins coordinating deubiquitination and acetylation-driven ubiquitination. *Sci Signal* **3**, ra80 (2010).
12. Karagianni, P., Amazit, L., Qin, J. & Wong, J. ICBP90, a Novel Methyl K9 H3 Binding Protein Linking Protein Ubiquitination with Heterochromatin Formation. *Mol. Cell. Biol.* **28**, 705-717 (2008).
13. Kraft, E., Bostick, M., Jacobsen, S.E. & Callis, J. ORTH/VIM proteins that regulate DNA methylation are functional ubiquitin E3 ligases. *Plant J* **56**, 704-715 (2008).
14. Barrès, R. et al. Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. *Cell Metab* **10**, 189-198 (2009).
15. Lister, R. et al. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* **462**, 315-322 (2009).
16. Stroud, H. et al. Comprehensive analysis of silencing mutants reveals complex regulation of the Arabidopsis methylome. *Cell* **152**, 352-364 (2013).
17. Qian, C. et al. Structure and hemimethylated CpG binding of the SRA domain from human UHRF1. *J Biol Chem* **283**, 34490-34494 (2008).
18. Wang, L. et al. A low-cost library construction protocol and data analysis pipeline for Illumina-based strand-specific multiplex RNA-seq. *PLoS ONE* (2011). doi:10.1371:journal.pone.0026426.
19. Trapnell, C. et al. TopHat: discovering splice junctions with RNA-seq. *Bioinformatics* (2009) doi:10.1093/bioinformatics/btp120.
20. Anders, S. and Huber, W. Differential expression analysis for sequence count data. *Genome Biol* (2010) doi:10.1186/gb-2010-11-10-r106.